Bacteriophage Can Prevent Encrustation and Blockage of Urinary Catheters by Proteus mirabilis.

Nzakiwanayo, J., Hanin, A., Alves, D. R., McCutcheon, B., Dedi, C., Salvage, J., Knox, K., Stewart, B., Metcalfe, A., Clark, J., Gilmore, B., Gahan, C. M. C., Jenkins, T. A., & Jones, B. V. (2016). Bacteriophage Can Prevent Encrustation and Blockage of Urinary Catheters by Proteus mirabilis. *Antimicrobial Agents and Chemotherapy*, *60*(3), 1530-1536. https://doi.org/10.1128/AAC.02685-15

Published in:
Antimicrobial Agents and Chemotherapy

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
Link to publication record in Queen's University Belfast Research Portal

Title: Bacteriophage can prevent encrustation and blockage of urinary catheters by *Proteus mirabilis*

Running title: Bacteriophage can prevent catheter blockage

Jonathan Nzakizwanayo[a], Aurélie Hanin[b], Diana R. Alves[a,c,d], Benjamin McCutcheon[a], Cinzia Dedi[a], Jonathan Salvage[a], Karen Knox[e], Bruce Stewart[e], Anthony Metcalfe[a,c], Jason Clark[f], Brendan Gilmore[g], Cormac M. C. Gahan[b], A. Toby A. Jenkins[h], Brian V. Jones[a,d]#

[a] School of Pharmacy and Biomolecular Sciences, University of Brighton, Brighton, East Sussex, United Kingdom.

[b] Alimentary Pharmabiotic Centre and School of Microbiology, University College Cork, Ireland.

[c] Blond McIndoe Research Foundation, Queen Victoria Hospital, Holtye Road, East Grinstead, United Kingdom.

[d] Queen Victoria Hospital NHS Foundation Trust, Holtye Road, East Grinstead, United Kingdom.

[e] East Surrey Hospital, Surrey and Sussex NHS Healthcare Trust, Redhill, Surrey, United Kingdom.

[f] Novolytics Ltd, Daresbury Science and Innovation Campus, Warrington, United Kingdom.

[g] School of Pharmacy, Queens University Belfast, Belfast, United Kingdom.

[h] Department of Chemistry, University of Bath, Bath, United Kingdom.

#Corresponding Author: Brian V. Jones, e-mail: b.v.jones@brighton.ac.uk

ABSTRACT

Proteus mirabilis forms dense crystalline biofilms on catheter surfaces that occlude urine flow leading to serious clinical complications in long-term catheterised patients, but there are presently no truly effective approaches to control catheter blockage by this organism. This study evaluated the potential for bacteriophage therapy to control *P. mirabilis* infection and prevent catheter blockage. Representative *in vitro* models of the catheterised urinary tract, simulating a complete closed drainage system as used in clinical practice, were employed to evaluate the performance of phage therapy in preventing blockage. Models mimicking either an established infection, or early colonisation of the catheterised urinary tract, were treated with a single dose of a 3 phage cocktail, and the impact on time taken for catheters to block, as well as levels of crystalline biofilm formation, were measured. In models of established infection phage treatment significantly increased time taken for catheters to block (~3-fold) compared to untreated controls. However, in models simulating early stage infection phage treatment eradicated *P. mirabilis* and prevented blockage entirely. Analysis of catheters from models of established infection, 10 hours after phage application, demonstrated that phage significantly reduced crystalline biofilm formation, but did not significantly reduce the level of planktonic cells in the residual "bladder" urine. Taken together, these results show that bacteriophage constitute a promising strategy for the prevention of catheter blockage, but that methods to deliver phage in sufficient numbers and within a key therapeutic window (early infection) will also be important to the successful application of phage to this problem.

INTRODUCTION

A frequent complication associated with long-term urethral catheterisation is encrustation and blockage of catheters due to infection with *Proteus mirabilis,* which can be isolated from around 45% of catheter associated urinary tract infections (CAUTI) (1, 2). Blockage stems from the ability of *P. mirabilis* to form dense biofilms on catheter surfaces, and the production of a potent urease enzyme which generates ammonia through hydrolysis of urea (1, 3, 4). Ammonia production elevates urinary pH causing the precipitation of calcium and magnesium phosphates, and the subsequent formation of crystals which become trapped within developing biofilms (1, 5). Once embedded in the biofilm, crystal growth is stabilised and enhanced by the biofilm matrix (6, 7). As this process continues the biofilm gradually becomes mineralised, leading to development of extensive crystalline biofilm structures which ultimately block catheters (1, 5). If unnoticed, blockage can lead to reflux of infected urine to the upper urinary tract, and the onset of serious clinical complications including pyelonephritis, septicaemia, and shock (1, 8).

Although catheters containing antimicrobial coatings are currently available, their efficacy in preventing infection during even short-term use remains questionable, and all available catheter types remain susceptible to *P. mirabilis* encrustation and blockage (9, 10). *P. mirabilis* is also extremely difficult to eliminate once established in the catheterised urinary tract and often responds poorly to conventional antibiotic therapy. It can persist despite multiple catheter changes or periods without catheterisation, and causes chronic infection and blockage in many patients (8, 9, 11). There are presently no truly effective strategies for the control of *P. mirabilis* CAUTI and associated blockage, and the development of new approaches is urgently required. The aim of this study was to determine if bacteriophage (phage) therapy

may constitute a viable approach to the prevention of catheter encrustation and blockage.

MATERIALS AND METHODS

Bacterial strains, media, routine culture. Clinical isolates of *P. mirabilis* (designated RS1 and RS3) used in this study were obtained from the Royal Sussex County Hospital, and all were derived from urinary tract infections. All chemicals, reagents and growth media were obtained either from Fisher Scientific UK, Oxoid UK, or Sigma UK unless otherwise stated. Bacteria were routinely cultured in Lysogeny-Broth Derivative Broth (LBDB) medium (5 g/l Yeast Extract, 10 g/l Vegetable peptone Nº1, 10 g/l Sodium Chloride) at 37°C with shaking, or on LBDB solidified by the addition of 15 g/l Technical agar (LBDA). Soft agar overlays, used for phage enrichments, purification and enumeration, were derived from LBDA (S-LBDA) and contained 5 g/l Yeast extract, 10 g/l Vegetable peptone Nº1, and 5.75 g/l Technical agar, and was kept molten at 45ºC for use in agar overlays. The artificial urine (AU) medium previously described by Stickler *et al.* (12), was initially prepared as a 5X concentrated stock solution containing sodium disulfate (11.5 g/l), magnesium chloride (hexahydrate) (3.25 g/l), sodium chloride (23 g/l), trisodium citrate (3.25 g/l), sodium oxalate (0.1 g/l), potassium dihydrogen orthophosphate (14 g/l), potassium chloride (8 g/l), ammonium chloride (5 g/l), calcium chloride dihydrate (3.25 g/l), urea (125 g/l), gelatin (25 g/l), and tryptone soya broth (5 g/l). Stock solutions of urea and calcium chloride dihydrate were sterilised separately by membrane filtration (0.45 µm; Sartorius, United Kingdom) while other components were sterilised by autoclaving. For use in bladder models all components were combined and diluted to 1X strength using sterile deionised water, with the final pH adjusted to 6.1.

103

104 **Phage isolation and purification.** Phages were isolated from sewage collected from
105 wastewater treatment plants in the UK (Anglian water, Luton area). For initial
106 enrichments of *P. mirabilis* phage 387.5 ml of LBDB was mixed with 100 ml of
107 sewage, and inoculated with 2.5 ml of host growing cultures of *P. mirabilis*.
108 Enrichments were incubated statically overnight at 37ºC and the following day 10 ml
109 aliquots were recovered, centrifuged (3000 X *g* for 30 min), and supernatants filtered
110 into fresh sterile tubes using 0.22 µm pore syringe filters (Sartorius, United Kingdom).
111 100 µl of filtered enrichment was mixed with 100 µl of a *P. mirabilis* exponential
112 phase growing culture to be used as phage host (either clinical isolate RS1 or RS3),
113 combined with 3 ml molten S-LBDA, swirled gently, and immediately poured over the
114 surface of an LBDA plate. Plates were incubated at 37ºC for 18-20 h. Phage
115 replication was identified by zones of lysis (plaques) in the confluent bacterial growth
116 within S-LBDA overlays. To isolate and purify distinct phage individual plaques were
117 picked off using Pasteur pipettes, and resuspended in 300 µl SM Buffer (100 mM
118 NaCl, 10 mM $MgSO_4.7H_2O$, 50 mM Tris-HCl pH 7.5, 0.01% gelatine). The resulting
119 phage suspensions were serially diluted in SM Buffer (10^{-3} to 10^{-6}), and dilutions used
120 to repeat agar overlays with host strains of *P. mirabilis* used in initial isolation. To
121 ensure clonality of phage types, this process was repeated a further 5 times until
122 bacterial lawns showed homogeneity of plaque morphology. Finally, an individual
123 plaque was picked off and resuspended in SM buffer for use in subsequent
124 experiments. These final clonal phage suspensions were stored at 4ºC until required.

125

126 **Preparation of high titre phage stocks.** Phage were propagated on *P. mirabilis*
127 RS1 host and high titre stocks obtained. Briefly, 100 µl of phage suspension and 100
128 µl of host growing culture were mixed, combined with 3 ml of molten S-LBDA, swirled

gently and poured onto agar plates. After a static overnight incubation at 37°C, plates displaying confluent lysis were selected and 3 ml of SM buffer supplemented with 2% (v/v) chloroform (to lyse remaining bacterial cells and maximize yield) were added before incubation at 37°C for 4 h. High-titre phage solution was removed from the plates, centrifuged (8,000 X *g* for 10 min) to remove cell debris, and then filter-sterilised (pore size, 0.22 µm) and stored at 4°C.

***In vitro* bladder models.** *In vitro* bladder models, originally described by Stickler *et al.* (12), were set up and operated as described previously (13). The key features of models are illustrated in **Figure 1**, and consist of a double-walled glass chamber (the bladder) maintained at 37°C by a water jacket supplied from a circulating water bath. Size 14 French all-silicone Foley catheters (Bard, United Kingdom) were used in all experiments, and are inserted into the "bladder" *via* an outlet in the base of the glass chamber, before retention balloons are inflated with 10 ml sterile water. Catheters were subsequently attached to a drainage bag to form a sterile closed drainage system, and AU medium supplied at a constant flow rate of 0.75 ml /min. *P. mirabilis* RS1 cell suspensions were inoculated directly into the residual bladder urine at either 10^{10} cfu or 10^{3} cfu representing late sage or early stage infection respectively, and flow suspended for 1h to permit cells to establish within the system. 45 min after bacterial inoculation, test models were treated with a single dose of 3 x 10^{10} pfu of a 3 phage cocktail (1:1:1, 10^{10} pfu of each phage) in a volume of 1 ml, and flow restored 15 min later. The numbers of viable cells present in the residual bladder medium were enumerated at the start and end of experiments, and pH was also measured at the start and end of experiments by sampling the medium in the "bladder."

Quantification of crystalline biofilm formation on catheter sections. To measure the levels of crystalline biofilm formation and catheter encrustation in control and phage treated models, the amount of calcium present on catheter sections removed from bladder models run for a set time (10 h) was quantified by flame photometry, described previously (13). Briefly, 1 cm catheter sections were submerged in 2 ml of an ammonium oxalate and oxalic acid solution (95% and 5% vol/vol respectively from 0.1 M stock solutions), subject to vigorous mixing for 3 min, then incubated at room temperature for 30 min. Catheter sections were then removed, the remaining mixture centrifuged (3000 X *g* for 10 min) and the supernatant discarded. Pellets were resuspended in 5 ml perchloric acid (0.05 M), samples mixed thoroughly, centrifuged (3000 X *g* for 2 min), and supernatants recovered. Levels of calcium dissolved in supernatants were determined using a flame photometer (Corning, Flame Photometer 410), calibrated using calcium standards at 100, 75, 50 and 25 ppm.

SEM of catheter cross sections. The thickness of biofilms and extent of encrustation on catheters recovered from timed models was visualised by SEM. Catheters were sectioned as shown in Fig. 4A, and mounted directly onto aluminium stubs using Leit adhesive carbon tabs (Agar Scientific, Stansted, United Kingdom). Mounted sections were stored overnight in a desiccator at RT then sputter coated with platinum using a Quorum Q150T ES system (Quorum Technologies, United Kingdom) and viewed using a Zeiss Evo LS15 microscope under high vacuum at an accelerating (EHT) voltage of 5 Kv and using a 5Q-BSD.

Transmission electron microscopy of bacteriophage. Purified phage particles (10^9 pfu/ml) were immobilised on a 200 mesh Formvar/Carbon copper electron microscope grids (Agar Scientific, UK), and negatively stained with 2%

phosphotungstic acid (pH 7.4) (Sigma, UK). Phage were imaged by FEG-STEM using a Zeiss SIGMA FEG-SEM microscope at 20 Kv accelerating voltage, 20µm aperture, and 2.7 mm working distance.

Analysis of Data. All statistical analysis was performed using Prism 6.0c For Mac OS X (Graphpad Software inc. USA; www.graphpad.com). Data was analysed using either Student's t-test, or ANOVA with the Bonferroni multiple comparisons test.

RESULTS

Bacteriophage isolation and characterisation. Three lytic phage, designated ΦRS1-PmA, ΦRS1-PmB, and ΦRS3-PmA, were isolated from wastewater through enrichments against clinical isolates of *P. mirabilis*. These phage showed distinct but overlapping host ranges (against a panel of 51 clinical isolates; data not shown) and differences in plaque morphology (**Fig. 2**). All were observed to generate halos around plaques, indicative of polysaccharide depolymerise activity, and were classified as members of the *Podoviridiae* based on TEM observations of capsid morphology (**Fig. 2**). All three phage were included in a "cocktail" in equal proportions (1:1:1) for evaluation of phage therapy in representative models of the catheterised urinary tract.

Effect of phage therapy on catheter blockage. Initial experiments replicated a worst-case scenario in which phage were used to treat an established infection (10^{10} cfu *P. mirabilis* in bladder models). Under these conditions a single "dose" of the phage cocktail (10^{10} pfu, MOI 1:1 phage:bacteria) significantly extended the time taken for catheters to block (~3 fold) (**Fig. 3**). Because interventions affecting blockage under these highly challenging conditions are likely to have greater impact

when applied earlier in the infection process, we next evaluated the impact of the same phage "dose" in experiments replicating the early stages of infection (10^3 cfu *P. mirabilis,* MOI $1:10^{-7}$ phage:bacteria). Under these conditions, the phage cocktail completely prevented catheter blockage and eradicated infection, with models draining freely for > 8 days until media reserves were exhausted (**Fig. 3**). In contrast, catheters in corresponding control models developed substantial encrustation, and became blocked after ~2 days (**Fig. 3**).

Effect of phage treatment on crystalline biofilm formation. To specifically evaluate the impact of phage treatment on crystalline biofilm formation, models of late stage infection were deactivated after 10 h, and levels of calcium on catheter sections quantified. This demonstrated that phage treatment significantly reduced levels of encrustation (**Fig. 4A**). These data were supported by direct SEM visualisation of catheter sections, which showed sections from models treated with phage to be devoid of visible crystalline deposits. This was in stark contrast to catheter sections from untreated models, which exhibited prominent encrustations (**Fig. 4B**). While these observations corresponded with a significant reduction in pH in treated models, the number of viable planktonic cells in residual urine from test or control models was not found to be significantly different (**Fig. 4C,D**).

DISCUSSION

Here we demonstrate the potential for bacteriophage to constitute an effective countermeasure for one of the most common and serious complications of long-term urethral catheterisation: encrustation and blockage. Our findings are congruent with previous studies examining the potential to control biofilm formation on urinary catheters using phage, where a reduction in biofilm formation by *P. mirabilis,*

Escherichia coli, Pseudomonas aeruginosa and *Staphylococcus epidermidis* has been reported when catheter sections were pretreated with phage suspensions (14, 15, 16). More recently, Lehman and Donlan (17) have described phage pretreatment for control of mixed species biofilm formation (*P. aeruginosa* & *P. mirabilis*), and also evaluated encrustation of catheter sections. However, these previous studies were able to show a reduction, rather than complete prevention, of biofilm formation by uropathogens tested, and where *P. mirabilis* was used phage did not fully prevent encrustation (14, 16, 17).

In contrast our data highlight the potential for a more dramatic impact of phage therapy in preventing blockage and resolution of *P. mirabilis* infection. Although specific attributes of the phage used in this study may be important to the outcome of bladder model experiments reported here, the differences in phage performance noted between this and other studies most likely relates to the high titres of phage achieved in bladder models, and delivery directly to residual bladder urine. In contrast, previous studies targeting *P. mirabilis* or other uropathogens have focused on the pretreatment of catheter sections with phage suspensions prior to use in models of biofilm formation (14, 15, 16, 17). As a result, the final titres of phage tested in these systems (and resulting MOIs established) was unclear, but likely to be substantially lower than those obtained in our models.

In addition, previous evaluations of phage therapy for CAUTI have mainly been designed to evaluate the ability of phage to reduce biofilm formation in general, rather than prevent catheter blockage specifically. In this context, the focus of our study on blockage as a specific therapeutic end point, and the evaluation of phage using a full closed drainage system in the bladder model system is also a key

difference. This model provides an excellent representation of the catheterised urinary tract and assessment of phage therapy in this setting.

In contrast, previous studies have used either simple static models of biofilm formation on catheter sections (14), or deployed models that do not use whole intact catheters or fully replicate the closed drainage system (15, 16, 17). While such models of infection clearly provide a useful and valid insight into the potential of phage therapy for CAUTI, and the control of bacterial biofilms in this setting, the encrustation and blockage of catheters is also governed by physical characteristics of distinct regions of catheters and the physicochemical forces that develop in the closed drainage system (1).

Most notably, blockage typically occurs around the catheter eye-hole and the first few centimetres of the catheter, which provide more irregular surface topologies [arising from the manufacturing process] that are particularly supportive of bacterial colonisation, and are continually exposed to the sump of infected residual urine that accumulates in the bladder (1,12,13). Therefore, the bladder model system provides a particularly robust evaluation of interventions aimed at prevention of blockage and encrustation, and the use of this system strengthens the observations reported here around the potential of phage to prevent catheter blockage.

Nevertheless, it is notable that phage were only able to fully prevent blockage when used in models of early stage infection. The simplest explanation for failure to prevent blockage in simulations of established infection is that the dose of phage used was insufficient to deal with the dense *P. mirabilis* population, and under MOIs established *P. mirabilis* growth and crystalline biofilm formation simply outstripped the

capacity of phage to eliminate infection. This may have been compounded by factors such as wash out of phage from model systems during the course of experiments, as well as the rapid elevation of urinary pH in models of late stage infection, which may reach pH 8 ~2-3 h after model activation.

Conversely, the recovery of phage from models of early infection 8 days after model activation (albeit at low levels, ~20 pfu/ml), despite an apparent absence of host bacteria for the majority of this time and the far longer duration of these experiments compared with models of established infection, argues against washout as a significant factor. Under conditions of high pH it is possible phage may be inactivated or their ability to infect host cells reduced, leading to eventual therapeutic failure. Previous evaluations of *P. mirabilis* phage have indicated that these remain active even under conditions of high pH (17). Our own evaluation of specific phage used here confirms these remain capable of infection after exposure to high pH (data not shown), but the possibility that alterations to cell surface properties protects against infection with these specific phage at high pH cannot be excluded.

Alternatively, the failure of phage to prevent blockage in late stage infection may be explained by the development of resistance to the phage used, and this has been observed in other studies of phage therapy for CAUTI over a similar time frame (16). Although the use of a three phage cocktail should guard against resistance, the phage used here have similar host range profiles, are all members of the *Podoviridae* family, and are yet to be characterised genetically. It is therefore possible that they constitute closely related phage types with comparable mechanisms of attachment and infection. This could allow the same mutation(s) in host bacteria to afford resistance to all three. In this context it is notable also that many key surface

structures of *P. mirabilis* that may be receptors for phage attachment are subject to phase variable gene expression (18), and it is therefore not unlikely that a small proportion of a given *P. mirabilis* population may be naturally immune to particular phage types, and selected for during phage treatment.

Despite this, there is clear potential to address the issue of resistance by ensuring selection of phage binding distinct cell surface structures, and generating a greater understanding of the mechanisms underpinning phage:host interaction in *P. mirabilis,* particularly under conditions encountered in the catheterised urinary tract. Furthermore, the high MOIs achieved in models of early infection also raises the potential for the induction of lysis from without (LO), which could also explain the differences in efficacy of phage treatment in the two infection scenarios modelled. The induction of LO could be highly advantageous in control of *P. mirabilis* CAUTI and subsequent studies should explore if *P. mirabilis* phage used here can induce LO, and the applications of this to control of CAUTI.

It was also clear from timed bladder model experiments that phage treatment significantly reduced levels of crystalline biofilm formation in models of established infection. Intriguingly, this work also suggests that the impact of phage treatment on *P. mirabilis* crystalline biofilm formation may not be solely attributable to a reduction in the number of planktonic cells available to participate in biofilm formation, since no statistically significant differences were observed in the number of viable planktonic cells in residual urine from test or control models at the 10h time point.

The putative polysaccharide depolymerase (PD) activity exhibited by the phage used here [based on halo production around plaques (19); **Fig 2**], may be important in this

regard. These enzymes, expressed on the surface of phage capsids or produced by host cells during phage replication, are believed to facilitate phage attack on biofilm communities by enabling phage penetration of the exopolymeric matrix (19, 20). The bioengineering of phage T7 has already demonstrated the potential utility of PD expressing phage in biofilm dispersal (20), and it is possible any PD activity of phage used in this study may contribute to their ability to reduce crystalline biofilm formation and encrustation, independent of cell lysis.

This highlights an additional feature of *P. mirabilis* phage that may be investigated further from the perspective of developing more broadly applicable anti-biofilm strategies. In the context of CAUTI, greater insights into the ability of phage to access biofilm associated cells could improve activity not only against mature biofilms, but perhaps more importantly multi-species biofilms. Challenges to the efficacy of phage therapy posed by multi-species biofilms would stem not only from the relatively narrow spectrum of activity of most phage, but also the possibility that mechanisms used by phage to access host bacterial cells within biofilms (such as PD enzymes), may be undermined by the chimeric EPS generated by multi-species biofilms (19).

Although recent work does indicate the potential to tackle multi-species biofilms with phage therapy (17), it would seem the more detailed study of phage:biofilm interactions and elucidation of associated mechanisms, coupled with the powerful approach of phage genome engineering, holds much potential for enhancing the efficacy of phage therapy in this regard. Alternatively, the prophylactic administration of phage active against key pathogens such as *P. mirabilis*, should also serve to offset issues associated with access to target cells in multi-species or even single species biofilms once these become established. Furthermore, there is also

considerable scope to combine phage therapy with other approaches to control infection such as antibiotics or other antimicrobial agents to enhance efficacy further.

In summary, the current study supports the potential efficacy of phage therapy in control of CAUTI, and in particular blockage caused by *P. mirabilis*. Although there is a clear need for further fundamental research into phage:host interactions and the ability of phage to control CAUTI to progress this approach, our work also suggests a major factor in the successful use of phage therapy in this setting will be the parallel development of strategies to deliver sufficient numbers of phage within the most effective therapeutic window (e.g. early stage infection for *P. mirabilis* CAUTI).

ACKNOWLEDGEMENTS

We wish to thank Joseph Hawthorn, Rowena Berterelli, and Heather Catty, for excellent technical support, and Dr Caroline Jones for constructive comments and criticism.

AUTHOUR CONTRIBUTIONS:

BVJ, and CMCG conceived and designed the study. JN, AH, DRA, BM, CD, JS conducted the experiments. BVJ, ATAJ, BG and CMCG directed the research. All authors contributed to analysis and interpretation of data. BVJ wrote the manuscript and all authors edited the manuscript.

CONFLICT OF INTEREST

JC is an employee of Novolytics Ltd that develops commercial bacteriophage products. JC provided expert advice and scientific support, but Novolytics Ltd provided no funding for the study and had no role in study design, interpretation of data, manuscript preparation, or decision to publish. The study funders also had no role in study design, data collection

and analysis, decision to publish, or preparation of the manuscript.

FUNDING

This study was primarily funded by the Queen Victoria Hospital NHS Foundation Trust, and the Queen Victoria Hospital Charitable Trust. DRA is supported by the Queen Victoria Hospital NHS Foundation Trust, the Blond McIndoe Research Foundation, and the University of Brighton. JN is also supported by the Dunhill Medical Trust (R394/1114 awarded to BVJ).

REFERENCES

1. Stickler DJ. Bacterial biofilms in patients with indwelling urinary catheters. 2008. Nat Clin Pract Urol **5**:598–608.

2. Mobley HLT. 1996. Virulence of *Proteus mirabilis*, p. 245–265. *In* Mobley HLT, Warren JW (ed), Urinary tract infections, molecular pathogenesis and clinical management, Washington, DC: American Society for Microbiology.

3. Griffith DP, Musher DM, Itin C. 1976. Urease. The primary cause of infection-induced urinary stones. Invest Urol **13**:346–350.

4. Jones BD, Mobley HL. 1987. Genetic and biochemical diversity of ureases of *Proteus, Providencia,* and *Morganella* species isolated from urinary tract infection. Infect Immun **55**:2198–2203.

5. Stickler D, Ganderton L, King J, Nettleton J, Winters C. 1993. *Proteus mirabilis* biofilms and the encrustation of urethral catheters. Urol Res **21**:407–411.

410 6. Clapham L, McLean RJC, Nickel JC, Downey J, Costerton JW. 1990. The influence of
411 bacteria on struvite crystal habit and its importance in urinary stone formation. J Cryst
412 Growth **104**:475–484.

413 7. Dumanski AJ, Hedelin H, Edin-Liljegren A, Beauchemin D, McLean RJ. 1994. Unique
414 ability of the *Proteus mirabilis* capsule to enhance mineral growth in infectious urinary
415 calculi. Infect Immun **62**:2998–3003.

416 8. Jacobsen SM, Stickler DJ, Mobley HLT, Shirtliff ME. 2008. Complicated catheter-
417 associated urinary tract infections due to *Escherichia coli* and *Proteus mirabilis*. Clin
418 Microbiol Rev **21**:26–59.

419 9. Morris NS, Stickler DJ, Winters C. 1997. Which indwelling urethral catheters resist
420 encrustation by *Proteus mirabilis* biofilms? Br J Urol **80:**58–63.

421 10. Morgan S, Rigby D, Stickler DJ. 2009 A study of the structure of the crystalline biofilms
422 that encrust and block silver Foley catheters. Urol Res **37:** 88–93.

423 11. Sabbuba NA, Mahenthiralingam E, Stickler DJ. 2003. Molecular epidemiology of
424 *Proteus mirabilis* infections of the catheterized urinary tract. J Clin Microbiol **41**:4961–
425 4965.

426 12. Stickler DJ, Morris NS, Winters C. 1999. Simple physical model to study formation and
427 physiology of biofilms on urethral catheters. Methods Enzymol **310**:494–501.

428 13. Holling N, Lednor D, Tsang S, Bissell A, Campbell L, Nzakizwanayo J, Dedi C,
429 Hawthorne JA, Hanlon G, Ogilvie LA, Salvage JP, Patel BA, Barnes LM, Jones BV.
430 2014. Elucidating the Genetic Basis of Crystalline Biofilm Formation in *Proteus*
431 *mirabilis*. Infect Immun **82**:1616–1626.

14. Carson L, Gorman SP, Gilmore BF. 2010. The use of lytic bacteriophages in the prevention and eradication of biofilms of *Proteus mirabilis* and *Escherichia coli*. FEMS Immunol Med Microbiol **59**:447–455.

15. Curtin JJ, Donlan RM. 2006. Using bacteriophages to reduce formation of catheter-associated biofilms by *Staphylococcus epidermidis*. Antimicrob Agents Chemother **50**:1268–1275.

16. Fu W, Forster T, Mayer O, Curtin JJ, Lehman SM, Donlan RM. 2010. Bacteriophage cocktail for the prevention of biofilm formation by *Pseudomonas aeruginosa* on catheters in an *in vitro* model system. Antimicrob Agents Chemother **54**:397–404.

17. Lehman SM, Donlan RM. 2015. Bacteriophage-mediated control of a two-species biofilm formed by microorganisms causing catheter-associated urinary tract infections in an *in vitro* urinary catheter model. Antimicrob Agents Chemother **59**:1127–1137.

18. Armbruster CE, Mobley HLT. 2012. Merging mythology and morphology: the multifaceted lifestyle of *Proteus mirabilis*. Nat Rev Microbiol **10**:743–754.

19. Sutherland IW, Hughes KA, Skillman LC, Tait K. 2004. The interaction of phage and biofilms. FEMS Microbiol Lett **232**:1–6.

20. Lu TK, Collins JJ. 2007. Dispersing biofilms with engineered enzymatic bacteriophage. Proc Natl Acad Sci U S A **104**:11197–11202.

454 **FIGURE LEGENDS**

455

456 **Figure 1: Illustration of *in vitro* bladder model system.** Models were set-up
457 according to specifications originally described by Stickler *et al* 1999 (12). **a)** Double
458 walled glass vessel representing the bladder. **b)** Foley catheter inserted into the
459 model and connected to drainage bag to form sterile closed drainage system**. c)**
460 Drainage tubing. **d)** Drainage bag collects urine outflow. **e)** Sterile urine/artificial urine
461 supplied to "bladder" via peristaltic pump at a constant flow rate. **f)** Water at 37 ˚C
462 circulated through outer bladder model chamber to maintain constant temperature.
463 Diagram is adapted from Holling *et al.* 2014 (13).

464

465 **Figure 2: Example of plaque morphology and capsid morphology in *P. mirabilis***
466 **bacteriophage evaluated in bladder models.** Images show plaque morphology for ΦRS1-
467 PmA, ΦRS1-PmB and ΦRS3-PmA generated on lawns of host strains used for isolation
468 (Strain RS1 for ΦRS1-PmA and ΦRS1-PmB, and strain RS3 for ΦRS3-PmA). Associated
469 transmission electron micrographs show structure of phage capsids, with morphology in all
470 cases congruent with members of the *Podoviridae* family.

471

472 **Figure 3: Impact of bacteriophage treatment on catheter blockage.** *In vitro*
473 models of the catheterised urinary tract replicating either a late stage heavy infection
474 (10^{10} cfu *P. mirabilis*), or early stage colonisation of the catheterised urinary tract (10^{3}
475 cfu *P. mirabilis*), were used to evaluate the impact of a single phage therapy
476 treatment on blockage and encrustation. For heavy infection test models were treated
477 with phage at an MOI 1:1 phage:bacteria. Test models replicating early stage
478 infection were treated with the same phage dose (MOI $1:10^{-7}$ phage:bacteria). Phage
479 treatments were applied 45 min after models were inoculated with *P. mirabilis*.

Models were run until catheters became blocked and urine ceased to accumulate in drainage bags, or media was exhausted. **A)** Time taken for catheters in control and phage treated models to become blocked, or for media to be exhausted. **B)**. pH of urine in residual bladder model media at end of experiments **C)** Enumeration of viable cells in residual urine in bladder models at the end of experiments. **All data:** Represent the means of 3 independent replicates. Error bars show the standard error of the mean. ** $P < 0.01$, **** $P < 0.0001$ Treated Vs Control in each model set-up. In models representing early infection and treated with phage no evidence of catheter blockage was observed and models were deactivated after 8 days when media was exhausted.

Figure 4: Impact of phage treatment on crystalline biofilm formation. Models replicating a established infection (10^{10} cfu *P. mirabilis*) were used to evaluate the impact of phage treatment on crystalline biofilm formation. Test models were treated with phage at an MOI 1:1 phage:bacteria (10^{10} pfu:10^{10} cfu), 1 h after model start. Both test and control models were deactivated after 10 h and levels of crystalline biofilm formation measured on descending sections. **A)** Schematic of urethral catheter showing sections subject to analysis in part B. **B)** Quantification of crystalline biofilm formation and encrustation on catheter sections (total calcium present on each catheter section examined). Images below the chart provide examples of SEM visualisation of catheter cross-sections, distal to section 1-3, and levels of encrustation. Bars on SEM images represent 200 µm. **C)** pH of urine in residual bladder model media at end of experiments **D)** Enumeration of viable cells in residual urine in bladder models at the end of experiments – no significant differences. **All data:** Data represent the mean of three replicate experiments, and error bars show

505 the standard error of the mean. ** P < 0.01, *** P < 0.001 control section Vs phage
506 treated model.